DIAGNOSIS OF SEPSIS USING THE LPS-BINDING MOIETY OF ALKALINE PHOSPHATASE

PRESENT STATE OF THE ART

Alkaline phosphatase (AP) is an ecto-enzyme bound via a glycosyl-phosphatidyl-inositol (GPI)-anchor to the plasma membrane of many cell types including endothelial and epithelial cells, osteoblasts, fibroblasts, and hepatocytes. The enzyme is also present in specific granula of neutrophils. Consequently, all organs contain abundant alkaline phosphatase activity. In addition, enzyme activity is also present in plasma.

Increased levels of AP activity in blood are found when bone formation is evident for instance during embryogenesis and childhood (3). Also hepatic diseases like cholestasis are characterized by high serum AP levels and an enhanced expression of AP activity upon plasma membranes of hepatocytes. This enzyme is therefore a generally accepted serum marker for the diagnosis of this disease (4). Release from neutrophilic granula after activation of this cell type may also contribute to the enhanced serum AP activity found during inflammatory processes. The physiological significance of this enzyme induction in liver diseases and inflammatory processes is unknown. A role of AP during the process of bone formation has been documented in vitro and in vivo.

Recent studies have demonstrated that AP is able to dephosphorylate endotoxin or lipopolysaccharide (LPS). LPS is a constituent of the cell wall of Gram-negative bacteria and it is very toxic to man and most animal species. It induces fulminant inflammatory reactions which may be life threatening when these responses occur within the bloodstream. This disease is referred to as 'sepsis' or 'systemic inflammatory response syndrome (SIRS)' and is lethal in approximately 35% of all cases. LPS may enter the bloodstream after bacterial infections, during hepatic diseases or when intestinal permeability is enhanced for instance during or after ischemic occurrences. High concentrations of LPS are present in the gastro-intestinal tract due to production by E. coli bacteria, Enterobacteriaceae, and many other bacterial species. Since virtually no LPS is found in plasma, the gastrointestinal tract obviously has an effective barrier. This barrier is characterized by a high AP expression similar to the blood brain barrier where neuro endothelial cells also express a high AP activity.

An enhanced intestinal permeability for LPS has been documented in patients with gastro-intestinal perforations, appendicitis or peritonitis but also in patients with alcoholic abuse, after traumatic events such as burns, major surgical operations to the gastro-intestinal tract and after sepsis. Most of these latter cases are characterized by post-ischemic reperfusion injury to the intestinal wall and concomitant release of an array of inflammatory mediators which affect the vascular permeability in the intestine thus causing leakage of LPS from the intestinal lumen into the vena porta. In normal conditions the liver plays an important role in the removal of LPS from the circulation, in particular the Kupffer cell is important in this respect. However, in many hepatic diseases this function is impaired which in turn may lead to endotoxemia.

Phosphate groups within the lipid A moiety of LPS determine several biological responses to this bacterial product. The lipid A moiety is the relatively constant part of this otherwise highly variable molecule. Generally, two phosphate groups are present upon this lipid A moiety in addition to a variety of phosphate groups within the polysaccharide tail. Whereas phosphate groups within the polysaccharide tail do not influence the inflammatory response upon LPS, the phosphate groups within the lipid A moiety are of major importance. It has been demonstrated that mono-phosphoryl lipid A is a weak inducer of macrophage responses and is non-toxic to chickens and rabbits, whereas native, di-phosphoryl lipid A is a potent activator of macrophages and lethal to most mammals.

The dephosphorylation of LPS by alkaline phosphatase may therefore be important in vivo and this creates new insights into the physiological role of this enzyme. This new insight may have many therapeutic implications. Document PCT/NL94/00189 describes the therapeutic application of alkaline phosphatase to treat all kinds of the clinical complications induced by LPS.

DESCRIPTION OF THE INVENTION

In summary, the examples show that serum AP activity declines in patients with established Gram-negative sepsis, whereas serum AP activity is unaffected or even is slightly enhanced in patients with established Gram-positive sepsis. This new finding may be explained by the binding of LPS to AP enzymes as demonstrated herein. This binding either may cause a change in the distribution of AP, thus reducing half-life in

serum or it may affect the enzyme activity directly. Based on all these observations, and considering the new insight on the role of AP in vivo as an LPS detoxifying enzyme (described in document PCT/NL94/00189), it can be concluded that AP has a binding site for LPS and that the binding of this ligand to the enzyme has important implications.

Early detection of endotoxemia in patients with liver failure, severe burns or other trauma's (e.g. after organ transplantations) is important but rather complicated. Reliable and sensitive methods which measure free LPS in serum and which take the endotoxin detoxifying capacity of serum also into account are not available yet. In addition, methods determining the amount of LPS in serum do not give any information about the preceding situation. This is particularly relevant in view of the short half-life of LPS in the bloodstream. Detection methods to measure the binding of LPS to AP as such are feasible and these methods can be applied for diagnostic purposes to discriminate between Gram-positive and Gram-negative sepsis or to assess the severity of the disease. The degree of occupation of the binding site appears to be an early marker for this disease. Thus a valuable new diagnostic tool is described here. In addition, based upon the insight that AP has a specific binding domain for LPS, new LPS binding systems can be developed that bind to this domain. These domains are able to remove LPS from fluid or tissue. ligands interacting with AP may also be used for the purification of AP from crude or partially purified biological sources (e.g. milk, culture media, tissue samples) Also systems applying the LPS binding domain of AP can also be used for therapeutic purposes.

Thus the invention describes the use of ligands to the LPS-binding site of alkaline phosphatase. Examples of such ligands are mono- or polyclonal antibodies against this ligand either in their natural occurring form or as Fab-fragments, single chains, or otherwise modified immunoglobulins. In addition, labeled LPS, lipid A, derivatives of both substances or products mimicking the tertiary structure of lipid A can be used as ligands for the LPS binding site in AP enzymes. It is possible to design such on the basis of computer modelling. The product can be produced synthetically using chemical means. There are references detailing production of lipid A like structures using chemical means (1,2). Use of recombinant DNA technology to engineer the required structure is also possible as is chemical modification of lipid A like structures. Measurement of the concentration of free LPS-binding domains on alkaline phosphatase for diagnostic purposes can be done using these products. Subsequently, this

concentration can be related to the total concentration of AP enzymes, or to the total enzyme activity in biological fluids which will yield additional information about the presence of LPS and the LPS-detoxifying capacity in biological fluids.

Thus the method acording to the invention consists of diagnosis of onset of endotoxemia or sepsis due to Gram negative bacterial infection said method comprising monitoring of the degree of AP occupancy of LPS binding sites on alkaline phosphatase in a sample of tissue or fluid derived from a patient, wherein the degree of AP occupancy is associated with presence or absence of Gram negative bacterial infection. Specifically such a method wherein the degree of AP occupancy of LPS binding sites on alkaline phosphatase in the sample is lower than that of an equivalent sample type of an individual free of Gram negative infection. By way of example the degree of AP occupancy of LPS binding sites on alkaline phosphatase in a sample or tissue or fluid derived from a patient, is monitored over a period of time, wherein a decline of the degree of AP occupancy indicates Gram negative bacterial infection. In such a case the degree of AP occupancy of LPS binding sites on alkaline phosphatase in the sample is determined and when onset of decline in the degree of AP occupancy is ascertained this indicates onset of Gram negative bacterial infection. It is also possible depending on the values determined that the degree of AP occupancy of LPS binding sites on alkaline phosphatase may also indicate a mixed infection of Gram negative and Gram positive bacteria. The test may indicate the onset of sepsis but may also identify those individuals at risk for sepsis e.g. those individuals at risk for sepsis e.g. persons with structural low AP levels in tissue or blood, due to a genetic defect or due to a chronic illness such as Rheumatoid arthritis, brain oedema, Alheimer, ischemic heart diseases or inflammatory bowel diseases. In addition the test may be used to assess (subclinical) infections in pregnant women. These subclinical infections may provoke the HELLP syndrome or pre eclampsia in the mother or affect the condition of the child by causing intraurine growth retardation of the foetus or even inducing pre-term delivery. As a consequence the test described here can be used to prevent pathology to the new born child. In a method according to the invention the sample to be determined can be subjected to binding with a ligand for the LPS binding site on alkaline phosphatase followed by determination of the degree of binding of the ligand. Any ligand with the required specificity can be used. The selection will depend on the type of sample to be tested and the degree of sensitivity to be reached. Quite specifically suitable ligands can be selected LPS, Lipid A, an LPS

binding site antibody against alkaline phosphatase, a Fab fragment with LPS binding site binding ability on alkaline phosphatase, a single chain fragment of an immunoglobulin having LPS binding site binding activity on alkaline phosphatase. Alternatively other ligands e.g. developed on the basis of computer modelling of the binding system of LPS and lipid A with alkaline phosphatase can also be used. The ligands may be naturally occurring molecules or synthetic molecules. The synthetic molecules may be produced using chemical or biochemical i.e. recombinant DNA technology methods. Preferably a method according to the invention employs an LPS binding site binding ligand with at least the affinity for the LPS binding site of alkaline phosphatase of LPS. Also a method according to the invention may suitably employ an LPS binding site binding ligand with at least the affinity for the LPS binding site of alkaline phosphatase of lipid A. The required degree of specificity and affinity can be ascertained using standard tests for comparing specificity and affinity of a compound for a substrate. In a method according to the invention the degree of AP occupancy of LPS binding sites on alkaline phosphatase can be determined by assessment of the dephosphorylating capacity of alkaline phosphatase in the sample. In such a method the ratio of dephosphorylating alkaline phosphatase to non dephosphorylating alkaline phosphatase is determined. The ratio can be determined using the values obtained by assessment of total alkaline phosphatase activity using biochemical methods to determine dephosphorylating activity and by assessment of total amounts of alkaline phosphatase using antibodies and calculating the ratio of these values. There are numerous routes available to the skilled person to carry out such assessments. We refer by way of example to references 5 and 6. The content hereof is incorporated by reference. It will immediately be apparent what other alternative known methods can be applied to arrive at the required data.

The sample to be used in the method according to the invention can be a sample derived from a patient. Such a sample can be from blood or tissue. The sample must be a sample treated such that any alkaline phosphatase present is not removed prior to the assay. Such a sample can be selected from the group consisting of blood and tissue. The blood sample can for example be serum. The tissue sample is preferably other than bone as alkaline phosphatase levels are generally high in bone. The tissue that is suitable for example can be selected from liver and intestines. A biopt can be taken in a manner known per se. Blood and serum samples may also be prepared in the usual manner.

The method according to the invention can be carried out with a sample from a cholestasis free patient. The method according to the invention can also be carried out on a patient with cholestasis. The values determined for the degree of LPS binding can be compared to standard values of healthy and/or sick patients to ascertain the correct diagnosis. Alternatively the method according to the invention can comprise carrying out the assay described above in any of the described embodiments and in addition also comprising a further assay of a sample from the patient for another disease related to increase of alkaline phophatase activity such as cholestasis. The further assay must preferably then employ a method avoiding determination of alkaline phosphatase level. Numerous articles describing such alternative assays for alkaline phosphatasae associated diseases are available in articles covering said diseases and the content thereof is incorporated by reference. A suitable embodiment of the method according to the invention wherein the further assay is carried out is when the alkaline phosphatase assay according to the invention reveals no decline in AP occupancy of LPS binding sites of alkaline phosphatase. Suitably the method according to any of the embodiments of the invention comprises a method wherein the sample is taken from an individual at risk of Gram negative bacterial infection. The sample can for example be taken from an individual either both prior to and following trauma. It can also be taken shortly after having undergone trauma. Quite interesting cases of trauma concern surgery, burns and ischemic trauma. As hospitals form notorious sources of infection suitably the method according to the invention can comprise assessment of a sample taken from an individual during hospitalisation.

In a method according to the invention the sample can be taken a number of times over a period of time. The data can be compared with eachother thus revealing the level of AP occupancy over time. The values can however also be compared with a standard value for a healthy or sick individual. The values can also be correlated to various characteristics of the patient e.g. gender, age, body weight, tissue or fluid type to be tested. The method thus reveals whether the degree of AP occupancy is indicative of endotoxemia or sepsis. A method according to the invention can comprise taking samples over a period of time as long as the individual is at risk of infection i.e. during hospitalisation or post trauma recovery.

The invention also envisages kits for carrying out the diagnostic method according to the invention. Such a kit can comprise alkaline phosphatase LPS binding

site binding ligand and instructions for carrying out an assay according to any of the abovementioned embodiments. The kit can further comprise any additional components required for such assay e.g. detectable marker, buffer, containers. Also suitable as additions to the kit are comparative samples or data charts e.g. standard curves or data concerning relevant data of alkaline phosphatase values. A kit comprising alkaline phosphatase LPS binding site binding ligand for carrying out an assay according to any of the abovementioned embodiments and any additional component required for such assay being selected from the following group consisting of detectable marker, buffer, containers, comparative samples, data charts e.g. standard curves or data concerning relevant data of alkaline phosphatase values is also part of the invention. The ligands envisaged have been described in the embodiments of the method as disclosed above and in the claims and examples.

In addition the invention covers a method for removing LPS from tissue or fluid said method comprising contacting the LPS binding site of alkaline phosphatase with the tissue or fluid to be treated followed by separation of the LPS binding site and the tissue or fluid after the the LPS binding site has bound the LPS present in the fluid or tissue, with the proviso the ligand is neither alkaline phosphatase nor a derivative of alkaline phosphatase having dephosphorylating activity. Thus the LPS can be removed from the tissue or fluid. Clearly the binding must occur under circumstances that do not damage the fluid or tissue to be purified. Such conditions will be readily apparent to the skilled person used to dealing with such tissue or fluid. By way of example the LPS-binding domain of AP itself can be coupled to a non-soluble carrier to remove LPS from tissue or fluids. Suitable fluids to be treated are cell-culture media, liquids used for organ perfusion or preservation, blood, milk or any other therapeutic product administered to a person. Ligands which bind to the LPS binding site of AP may also be used for purification of AP itself from tissue or body fluids or other biological production systems.

The invention also covers a method for therapy of endotoxemia or sepsis said method comprising administration of a pharmaceutically effective amount of the LPS binding site of alkaline phosphatase in a systemically acceptable form with the proviso the ligand is neither alkaline phosphatase nor a derivative of alkaline phosphatase having dephosphorylating activity. Any conventional medicinal dosage form will suffice. The dosage regime to be followed will depend on the condition of the patient, gender,

age, body weight by way of example and the physician will be able to determine the best regime to apply. The LPS-binding domain as such or in the form of a derivative of alkaline phosphatase, said derivative not having dephosphorylating activity may be applied therapeutically to treat a patient with a systemic inflammatory response syndrome or to prevent endotoxemia in patients at risk for such a complication. Binding of LPS in blood to this particular domain may prevent the interaction of LPS with other endogenous receptors in serum and upon plasma membranes of endothelial cells or macrophages such as LPS-binding protein, CD-14 and scavenger receptors. As a consequence, the inflammatory response upon LPS will be attenuated.

The invention also covers other therapy and diagnostic applications of the ligands described in the description of the invention and a method of purification of AP itself using LPS or any AP binding derivative thereof i.e. the other ligands mentioned.

EXPERIMENTAL DATA

EXPERIMENT I

These reports prompted us to examine the serum levels of AP during endotoxemia. It is generally accepted that AP is a marker of liver damage, and LPS is known to induce directly or indirectly damage to this organ. An increase in serum AP activity in patients with sepsis has been frequently documented and was also expected in mice with endotoxemia. However, after intravenous administration of LPS from E.coli (strain 055:B5, Sigma Chemical Co, USA, 2.5 mg/kg b.w.) to Balb/c mice (male, 20-25 g) at t=0 a rapid and sharp decline in serum AP activity was observed as compared to mice receiving saline (fig. 1). A similar decline was found after intraperitoneal administration of E.coli bacteria (ATCC 25922, 2 x 109 CFU) to mice (fig. 1).

Evaluation of clinical data of patients with recurrent endotoxemia revealed also a rapid primary sharp decline in serum AP activity, followed by a strong increase in the following days. It is believed that the first sharp decline of AP is caused by LPS. This decline enables residual LPS to evoke its inflammatory action thereby activating the destructive responses seen in sepsis patients. Clinically this primary reaction of AP decline has hitherto not been observed. Therefore to enable a fast detection of sepsis fast detection of declining AP levels is of most importance. The method described in the present document enables the measurement of such a fast kinetic change in AP levels. The basis in this lies in the fact that free and thereby fully active AP in plasma declines rapidly upon binding to LPS. The secondary increase in serum AP levels seen in most

patients may either reflect liver damage or may be induced by interleukin 6 or other inflammatory mediators which attenuate the expression of AP activity as recent studies have pointed out.

EXPERIMENT II

We examined subsequently 64 patients in the intensive care unit suffering from sepsis. The pathogenic bacteria causing the sepsis syndrome were identified in all of these cases. Patients with endotoxemia (n=16) were compared with patients with Gram-positive bacterial infections (n=30), which lack LPS in serum. The patients had a variety of underlying diseases, but patients with cholestasis, which is associated with an increased AP excretion, were excluded. Blood samples were routinely assayed for AP during hospitalization. Changes in serum AP levels were related to day O, that is, the day of admission to the intensive care unit of the hospital. We calculated the change in serum AP activity, relative to day 0, before and after the blood samples became positive for bacteria or bacterial products (LPS). These studies demonstrate that a decline in serum AP activity is an early marker of sepsis: the decrease preceded the bacteremia (fig. 2). Moreover, the data show that Gram-negative bacterial infections can be discriminated from Gram-positive bacterial infections because patients with established Gram-negative infections displayed a decline in serum AP activity (mean slope= -21.5) whereas patients with a proven Gram-positive bacterial sepsis showed a slight increase (mean slope= 10.9) relative to day zero. Note that at day zero (admittance to Intensive Care) the actual value of serum AP may have been affected already. Patients with a mixed infection (both Gram-positive and Gram-negative, n= 18) showed an intermediate change in the serum AP levels (mean slope= 0.6). The rapid diagnosis of Gram-positive versus a Gram-negative bacterial infection is particularly important in acute situations where an immediate start an effective antibacterial therapy is essential. To date, this diagnosis can only be made after a successful culture of the pathogenic bacteria, which takes usually two days.

EXPERIMENT III

We hypothesized that the decline in serum AP levels was caused by an increased turn-over of the enzyme. We therefore examined whether a direct binding between LPS and AP enzymes occurred and whether this binding would influence the

enzyme activity. Placental AP (6.7 U/ml), isolated from fresh human placenta's was incubated with LPS from E.coli (strain 055:B5, Sigma Chemical co; 1 mg /ml) for 10 minutes at room temperature. Subsequently, AP was subjected to FPLC analysis. The FPLC was equipped with an anion-exchange column (mono-Q HR 5/5, Pharmacia, Sweden) and UV detection (280 nm). PlAP was injected in 0.2 M Tris/HCl buffer (pH 7.4) containing MgCl2 with or without LPS. Elution was performed in Tris/HCl buffer (pH 7.4) with a flow rate of 0.5 ml/min and a NaCl gradient ranging from 0.0 to 0.45 M in 30 minutes. Retention time was measured. The retention time of plAP samples containing no LPS was 20 minutes, whereas the retention time shifted to 18 minutes after co-incubation with LPS. The shorter retention time of plAP samples containing LPS reflects an decreased negative charge of the complex, which indicates binding of LPS to AP enzymes thus neutralizing charges within the proteins.

EXPERIMENT IV

To assess whether product inhibition occurred, which might explain the decline in serum AP activity, we tested the effect of unphosphorylated lipid A, which is the end-product of the enzyme reaction, upon AP activity. Formalin/macrodex-fixed cryostat sections (4 um) of the rat intestine, kidney and placenta were incubated with LPS, MgCl2 and lead-nitrate (Pb2NO3) in 0.2 M Tris/maleic acid buffer (pH 7.4) to demonstrate phosphatase activity according to standard procedures (K. Poelstra et al. Lab. Invest 76:319, 1997, Am J. Pathol. 151: 1163, 1997).

As documented before, rat intestinal and renal cryostat sections expressed abundant LPS dephosphorylating enzyme activity. LPS-dephosphorylating activity in the rat placenta was less abundant. The localization of this enzyme activity was similar to the distribution of AP activity in all three organs. However, pre-incubation of these cryostat sections with lipid A (Monophosphoryl lipid A from Salmonella minnesota R595, List Biological Laboratories Inc., Campbell, USA; 1 mg/ml) strongly attenuated intestinal and renal phosphatase activity when LPS was subsequently added as substrate. No phosphatase activity was detectable in placenta's pre-incubated with lipid A.

From these data is has become clear that AP upon binding to LPS is temporarily not available for dephosphorylation of additional LPS. This may be explained by relatively high affinity of LPS to the binding site of AP and vice versa.

LEGENDS TO THE FIGURES:

Figure 1:

Serum alkaline phosphatase levels in mice after administration of saline (i.v.), LPS (i.v.) or E.coli bacteria (i.p.) at t=0 (n=4 per group). The diamond signifies saline, the square signifies E coli, the triangle signifies LPS. t is set in hr along x axis and AP activity is represented in U/L on y-axis

Figure 2:

Changes in serum AP levels relative to the day of admission to the intensive care unit of the hospital, before and after the blood samples of patients became positive for bacteria or bacterial products (LPS). Patients were divided into two groups: patients with an established Gram-negative bacterial infection (n=16) and patients with an established Gram-positive bacterial infection (n=30). Patients with a mixed bacterial infection (n=18) or with SIRS without identification of the bacterium are not presented here. The dark symbol signifies Gram negative. The light symbol signifies Gram positive.

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